1   <u>Cancer Diagnosis and Therapy</u>

2

3   <u>Technical Field</u>

4   The invention relates to a novel oncofetal

5   glycoprotein which is expressed in certain tumours,

6   antibodies to the protein, and uses of the

7   antibodies in cancer diagnosis.

8

9   <u>Background Art</u>

10   The cancer phenotype typically displays loss of

11   differentiation, loss of proliferative control and

12   altered cell adhesion molecule expression. Cell

13   surface proteins have been shown to play an

14   important role in cell-cell interactions (eg NCAM),

15   cell-extra-cellular interactions (eg CD44) and cell

16   regulation (eg Notch signaling).

17

18   Some of these cell surface proteins have oncofetal

19   expression profiles and as such have been used as

1 tumour specific diagnostic markers (eg CEA). A

2 further use for antibodies specific for cell

3 surface proteins over expressed in cancer has been

4 in the treatment of cancer by

5 immunotherapy/radioimmunotherapy (eg Herceptin an

6 antibody recognizing HER2).

7

8 <u>Statements of Invention</u>

9 In one aspect, the invention relates to an isolated

10 nucleic acid sequence which comprises a sequence

11 selected from the group consisting of: Sequence ID

12 No.1, Sequence ID No, 2, and Sequence ID No. 3.

13 Typically, the nucleic acid sequence is a DNA

14 sequence. In one embodiment, the isolated nucleic

15 acid sequence consists of a sequence selected from

16 the group consisting of: Sequence ID No. 1, Sequence

17 ID No. 2 and Sequence ID No. 3.

18

19 The invention also relates to an isolated protein

20 encoded by the isolated nucleic acid sequences of

21 the invention, or a fragment or analogue therof.

22 Typically, the protein is a cell surface

23 glycoprotein. In one preferred embodiment, the

24 isolated protein is an oncofetal protein expressed

25 by an astrocytoma cell. Typically, the protein has a

26 molecular weight of approximately 200kda. In this

27 specification, the term "protein" should be

28 understood as including amino acid sequences which

29 would more generally be referred to a peptides.

30

31 In another aspect, the invention relates to an

32 antibody which binds specifically to the protein of

1   the invention and any other antibody that competes

2   directly or by stearic hindrance therewith for said

3   protein. Typically, the antibody is a monoclonal

4   antibody. In one embodiment, the antibody is a class

5   M immunoglobulin with a kappa-light chain.

6

7   In another aspect, the invention relates to a

8   fragment of the antibody of the invention, which

9   fragment binds specifically to the protein of the

10 invention.

11

12 In another aspect, the invention relates to a method

13 of producing an antibody to a protein comprising:

14

15 - innoculating an animal with a protein according

16   to the invention, wherein the protein elicits an

17   immune response in the animal to produce the

18   antibody; and

19

20 - isolating the antibody from the animal.

21

22 In one embodiment, the animal is innoculated with G-

23 CCM cells of ECACC deposit No. 86022702.

24

25 In a further aspect, the invention relates to a

26 process for producing a hybridoma, comprising the

27 step of innoculating a suitable subject with a

28 protein according to the invention, or an antigenic

29 fragment thereof, and fusing cells from the subject

30 with a myeloma cell to produce the hybridoma.

31 Typically, the subject is innoculated with G-CCM

32 cells of ECACC deposit No. 86022702.

1

2 In a further aspect, the invention relates to a

3 hybridoma cell obtainable according to the above

4 process. In one embodiment, the invention relates to

5 a hybridoma cell of, or derived from, ECACC Deposit

6 No. 03073001.

7

8 A deposit of hybridoma cells according to the

9 invention was made at the European Collection of

10 Cell Cultures on 30 July 2003 and accorded the

11 accession number ECACC 03073001.

12

13 In another aspect, the invention relates to a

14 monoclonal antibody obtainable from a hybridoma cell

15 of, or derived from, ECACC Deposit No. 03073001.

16

17 The invention also relates to a method of detecting

18 an astrocytoma cell in a sample of human cells,

19 which method comprises the step of contacting the

20 cell sample with an antibody of the invention, or a

21 fragment thereof, and detecting those cells which

22 have bound the antibody or fragment, wherein binding

23 of the antibody or the fragment to a cell is

24 indicative of an astrocytoma cell. Typically, the

25 antibody is a monoclonal antibody of the invention.

26

27 The invention also relates to a method of detecting

28 a primary breast carcinoma cell in a sample of human

29 cells, which method comprises the step of contacting

30 the cell sample with an antibody of the invention,

31 or a fragment thereof, and detecting those cells

32 which have bound the antibody or fragment, wherein

1   binding of the antibody or the fragment to a cell is
2   indicative of a primary breast carcinoma cell.
3   Typically, the antibody is a monoclonal antibody of
4   the invention.
5
6   The invention also relates to a diagnostic kit for
7   diagnosing the presence of a cell selected from the
8   group consisting of: astrocytoma cells; malignant
9   melanoma secondary tumour cells; and primary breast
10 carcinoma cells, the kit comprising an antibody
11 according to the invention, or a fragment thereof.
12 Typically, the antibody is a monoclonal antibody of
13 the invention. In one embodiment, the antibody of
14 the invention comprises a detectable label.
15 Alternatively, the kit comprises a secondary
16 antibody which specifically binds the antibody of
17 the invention, which secondary antibody comprises a
18 detectable label.
19
20 The invention also relates to a biological targeting
21 device comprising an antibody, typically a
22 monoclonal antibody, of the invention, or a fragment
23 thereof, and a therapuetic ligand.
24
25 The invention also relates to a therapeutic antibody
26 comprising an antibody, typically a monoclonal
27 antibody, of the invention, or a fragment thereof.
28
29 The invention also relates to a method of treating
30 cancer in an individual by inducing apoptosis in
31 cells in the individual which express a protein of
32 the invention, which method comprises a step of

1   treating an individual with an antibody of the
2   invention, or a fragment thereof. Typically, the
3   antibody is a monoclonal antibody. In one
4   embodiment, the cancer is selected from the group
5   consisting of: malignant astrocytomas ; malignant
6   melanoma secondary tumours; and primary breast
7   carcinomas. Typically, the antibody is humanised.
8
9   The invention also relates to a polynucleotide which
10   is anti-sense to at least a portion of an insolated
11   nucleic acid sequence of the invention. Typically,
12   the polynucleotide is anti-sense to all or part of a
13   transcription initiator site of the isolated nucleic
14   acid sequence of the invention. In one embodiment,
15   the anti-sense polynucleotide comprises, or consists
16   of, a sequence of Sequence ID No. 4.
17
18   The invention also relates to a method of treating
19   cancer in an individual by inducing apoptosis in
20   cells in the individual which express a protein of
21   the invention, which method comprises a step of
22   treating an individual with an anti-sense
23   polynucleotide of the invention. In one embodiment,
24   the cancer is selected from the group consisting of:
25   malignant astrocytomas; malignant melanoma secondary
26   tumours; and primary breast carcinomas. Methods of
27   delivery of anti-sense polynucleotides will be well-
28   known to those skilled in the art of gene therapy.
29
30   The monoclonal antibodies of the invention may be
31   the complete antibodies described herein, or
32   fragments thereof. That is, they may be any fragment

1 of a monoclonal antibody of the invention that
2 specifically recognises the protein of the
3 invention. Such fragments include Fab, F(ab')2,
4 Fab', etc. These fragments ban be prepared by
5 digestion with an enzyme such as papain, pepson,
6 ficin, or the like. The properties of the obtained
7 fragments can be confirmed in the same manner as
8 described herein.
9
10 The principle reason for the poor prognosis
11 associated with malignant astrocytomas is recurrence
12 due to invasion of surrounding brain parenchyma by
13 tumour cells with an invasive phenotype. This
14 phenotype displays loss of differentiation,
15 secretion of proteases and altered cell adhesion
16 molecule expression. As part of an investigation
17 into the mechanisms of astrocytoma invasion,
18 monoclonal antibodies (Mab) were raised against cell
19 surface proteins expressed by an anaplastic
20 astrocytoma cell line (G-CCM). One of the
21 antibodies produced (MQ1 Mab) recognizes a
22 previously undescribed cell surface glycoprotein
23 (MQ1). In vitro MQ1 protein expression was found on
24 astrocytomas and fetal astrocytes, with the level of
25 expression increasing with astrocytoma malignancy
26 and decreasing with fetal astrocyte maturity.
27 Immunohistochemistry on histologically normal and
28 neoplastic brain tissue demonstrated that MQ1
29 protein expression is restricted to astrocytomas
30 (n=52). Other primary brain tumours tested
31 (oligodendrogliomas, neurinomas, PNET, and
32 medulloblastomas) and normal brain cells, including

1   neurons, oligodendrocytes and endothelial cells were

2   MQ1 negative, thus indicating that the MQ1 proteins

3   have the expression pattern of oncofetal proteins.

4   Similarily a study looking at primary breast

5   carcinomas found 60% were MQ1 positive (n=228).

6   Surrounding normal tissue, fibrocystic disease and

7   fibroadenoma tissue were MQ1 negative. Malignant

8   melanoma secondary tumours to the brain were also

9   found to be strongly MQ1 positive.

10

11   A cDNA expression library was synthesized from G-CCM

12   mRNA and screened with the MQ1 antibody. Two

13   positive clones were isolated (Sequence ID No.s 1

14   and 2) and sequencing data demonstrated that both

15   have a high degree of homology with Jagged1, a human

16   Notch ligand which plays a role in differentiation

17   and determination of cell fate. The library was

18   rescreened with probes generated from the positive

19   clones and further homologous transcripts were

20   isolated including a possible Jagged1 splice variant

21   (Sequence ID No. 3). Northern blotting for a range

22   of cell lines with these probes revealed the

23   presence of two transcripts (approximately 3.5kb &

24   5.0kb). Subsequent protein studies

25   (immunocytochemistry, immunoblotting and co-

26   immunoprecipitation) indicate that the MQ1 protein

27   has a high degree of homology with, but is not

28   identical to, Jagged1.

29

30   This investigation has identified a novel oncofetal

31   glycoprotein with homology to Jagged1. Its tumour

32   specificity together with its potential role in

1   regulating cellular differentiation /apoptosis

2   suggest that it may be a valuable prognostic marker

3   and therapeutic target.

4

5   The invention will be more clearly understood from

6   the following description of some embodiments

7   thereof, given by way of example only, with

8   reference to the following Figures in which:

9

10 Fig.1A illustrates confocal microscopy of live G-CCM

11 cells immunolabelled with MQ1 showing recognition of

12 a cell surface epitope;

13

14 Fig. 1B illustrates confocal microscopy of

15 permeabilized G-CCM cells immunolabelled with MQ1

16 showing recognition of an intracellular epitope and

17 localisation of the antigen at areas of cell contact

18 on the cell surface;

19

20 Fig 2 shows a comparision of MQ1 expression, by

21 immunocytochemistry and flow cytometry, on a range

22 of fetal astrocyte cultures and astrocytoma cell

23 lines. A-C show immunocytochemistry on live cells of

24 a grade IV, grade III and 16 week gestation fetal

25 astrocytes respectively. D-F chow the corresponding

26 flow analysis with the same cells with the level of

27 MQ1 surface expression estimated as mean channel

28 fluorescence. G shows the results of the flow

29 analysis plotted as a graph. This demonstrates an

30 inverse correlation of cell surface MQ1 protein

31 expression with fetal astrocyte maturity and

32 correlation with astrocytoma grade;

1

2   Fig.3 shows immunohistochemistry displaying diffuse

3   MQ1 positivity throughout A) Grade I astrocytomas B)

4   Grade II astrocytomas C) Grade III astrocytomas & D)

5   focal positivity in grade IV astrocytoma cells

6   palisading an area of necrosis;

7

8   Fig.4 shows MQ1 immunocytochemistry showing A)

9   strong MQ1 positivity at the tumour front B) strong

10   MQ1 positivity in reactive astrocytes in adjacent

11   tissue C) GFAP positivity in reactive MS tissue D)

12   MQ1 negative reactive MS tissue;

13

14   Fig. 5 shows MQ1 immunohistochemistry of breast

15   carcinoma tissue showing A) strong MQ1 positivity in

16   invasive ductal carcinoma cells surrounded by MQ1

17   negative stroma B) strong MQ1 positivity in lobular

18   carcinoma surrounded by MQ1 negative stroma;

19

20   Fig.6 shows MQ1 immunocytochemistry of G-CCM cells

21   treated with (A) 0.1µm control oligo (B) 0.5µm

22   control oligo (C) 1.0µm control oligo (D) 0.1 µm

23   anti-sense MQ1 oligo (E)0.5µm anti-sense MQ1 oligo

24   and (F) 1.0 µm antisense oligo, showing that MQ1

25   anti-sense oligo knocks out MQ1 protein expression

26   at concentrations of 0.5 and 1.0 µm;

27

28   Fig. 7 shows an immunoblot indicating Parp cleavage

29   of oligo-treated G-CCM cells;

30

31   Fig. 8 shows immunocytochemistry (ICC) detection of

32   cleaved Caspase 3 following oligo treatment1; and

1

2   Fig. 9 shows G-CCM cells labelled with MQ1 antibody

3   by ICC, 24 hours post-treatment with control and

4   anti-sense oligonucleotides in which:

5

6   (A)   control oligo 0.1µM

7   (B)   control oligo 0.5µM

8   (C)   control oligo 1.0µM

9   (D)   Anti-sense oligo 0.1µM

10   (E)   Anti-sense oligo 0.5µM

11   (F)   Anti-sense oligo 0.1µM

12

13. MATERIALS AND METHODS

14

15   Materials

16

17   All cell culture reagents were obtained from Gibco

18   BRL (Paisley, UK) with the exception of the

19   hypoxanthine, aminopterin and thymidine (HAT) and

20   the hypoxanthine and thymidine (HT) that were

21   obtained from Sigma (Poole, Dorset, UK). The

22   secondary and negative control antibodies were

23   supplied by Dako (Bucks, UK). The PARP and Caspase3

24   antibodies were purchased from Sigma (Poole, Dorset,

25   UK) and the Protein-A Sepharose CL4B from Pharmacia

26   Biotech (Herts, UK). PTO linked oligonucleotides

27   were obtained from MWG-Biotech (Germany).

28

29   Cell culture

30

31   The CB109 cell line was established from a

32   glioblastoma multiforme [6] and was a gift from Dr

1   Claude Chauzy (Centre Henri Becquerel, Rouen,

2   France). The G-CCM cell line was derived from a

3   human anaplastic astrocytoma and was a gift from Dr

4   Ian Freshney (Department of Clinical Oncology,

5   University of Glasgow, UK). The G-CCM cell line is

6   commercially available from the European Collection

7   of Cell Cultures under Deposit No 86022702. The

8   fetal astrocyte cell cultures were a gift from Ms

9   Kim Martin (Department of Neuropathology, Institute

10 of Psychiatry, London, UK). The C6 cell line,

11 derived from a rat glioma , was obtained from Flow

12 Laboratories (Scotland, UK). The skin fibroblast

13 cell culture was initiated in our laboratory from a

14 surgical specimen obtained from the Neurological

15 Unit (Royal Victoria Hospital, Belfast, UK). The

16 remaining glioma cell lines were initiated in our

17 laboratory from surgical specimens received from the

18 Neurosurgical Unit (Royal Victoria Hospital,

19 Belfast, UK) and were used experimentally after 5-10

20 passages. Tumour grading follows the World Health

21 Organisation classification. Cell lines were

22 incubated at 37oC/5% CO2 in Dulbecco's modified

23 Eagle's medium (DMEM) containing 2mM glutamine, 10%

24 fetal calf serum (FCS), and phenol red. All cell

25 lines were tested for mycoplasma using Hoechst 33258

26 fluorescent dye and were found to be negative.

27

28 Monoclonal antibody production

29

30 Mabs were produced utilizing a standardized protocol

31 designed to promote a rapid predominantly IgG

32 response. In brief, a BALB/c mouse was inoculated

1   intra-peritoneally with 5x106 G-CCM cells in 1ml of
2   Freund's complete adjuvant. Similar doses
3   emulsified in Freund's incomplete adjuvant were
4   administrated 14 and 28 days later to boost the
5   immune response. Four days after the final booster
6   inoculation the mouse was killed, its spleen
7   aseptically removed and the splenocytes induced to
8   fuse with NSO myeloma cells (at a ratio 5:1) using
9   polyethylene glycol. The resulting fusion products
10 were suspended in a selective, HAT-supplemented,
11 growth medium (RPMI-1640 medium containing 10mM L-
12 glutamine, 1% sodium pyruvate, 100 iu/ml penicillin,
13 100□g/ml streptomycin and 20% Myoclone FCS) and
14 seeded into 96-well plates. The medium, from the
15 viable hybridomas produced, was screened by indirect
16 immunofluorescence against live and acetone-fixed G-
17 CCM cells. Those showing specific recognition were
18 recloned three times, to ensure monospecificity, in
19 HT-supplemented growth medium and stored in liquid
20 nitrogen. The hybridoma cell line MQ-1, which
21 produced an antibody recognizing a cell surface
22 antigen was propagated as an ascitic tumour in
23 BALB/c mice previously immunosuppressed with
24 Pristane. The ascitic fluids were collected,
25 centrifuged and frozen at -20oC until use.
26
27 The positively labelling Mabs were isotyped for
28 their class and light chains using a monoclonal
29 antibody isotyping kit.
30
31
32

1   Immunofluorescence

2

3   Hybridoma medium (neat) or ascites fluid (diluted

4   1:200 in PBS) was incubated with living cells, grown

5   to 90% confluence on coverslips, for 40 min at room

6   temperature (RT). After washing, the cells were ·

7   fixed in acetone at -20oC for 10 min followed by

8   rehydration in PBS and incubation with an FITC-

9   conjugated rabbit antimouse antibody (FITC-RAM) for

10   30 min at RT. After two further washes the cells

11   were mounted on a glass slide, in a drop of

12   Citifluor, and examined using a Zeiss

13   immunofluorescence microscope or a Biorad confocal

14   microscope. Incubations in PBS without primary

15   antibody were used as negative controls. The

16   fluorescent labelling of positive cells was

17   subjectively rated from low intensity (+) to high

18   intensity (++++).

19

20   Flow Cytometry

21

22   A preliminary study (results not shown) comparing

23   the expression of MQ-1 protein on cells removed

24   enzymatically (trypsin) and non-enzymatically (0.53

25   mM EDTA in PBS) from culture flasks, revealed that

26   the MQ1 protein epitope was trypsin-resistant.

27

28   Cultured cells were removed from the flasks by

29   trypsinization, counted and aliquoted into

30   centrifuge tubes at a concentration of 5x105 cells

31   per tube. Triplicate samples were incubated in

32   excess ascitic fluid in 200□l of serum free medium

1   containing 1% bovine serum albumin (SFM/BSA) for 40

2   min at RT with gentle agitation. Following 2

3   washes in SFM the cells were incubated in an FITC-

4   RAM antibody for 30 min at RT with gentle

5   agitation. The cells were then washed twice in SFM

6   and fixed in PBS containing 1% para-formaldehyde.

7   The samples were analysed within 48 hr of fixation,

8   using a Coulter EPICS Elite flow cytometer.

9   Negative controls were incubated with an antibody

10 raised against Aspergillus niger glucose oxidase, an

11 enzyme not present or inducible in mammalian cells.

12 The consistency of the mean channel fluorescence

13 measurements between sample batches was checked

14 using EPICS Immuno-Brite standards.

15

16 Immunohistochemistry

17

18 On receipt the tissue was fixed in 10% formalin

19 prior to routine embedding in paraffin wax using a

20 Tissue Tex VIP (Miles Scientific) automated

21 processor. The paraffin blocks were sectioned at a

22 thickness of 6mm and mounted onto 3-

23 aminopropyltriethoxysilane-coated slides. The

24 tissue sections for indirect immunohistochemistry

25 were processed using an avidin-biotin peroxidase

26 complex (ABC) method. The tissue was dewaxed in

27 xylene and rehydrated before endogenous peroxidase

28 activity was blocked by a 10min incubation in 3%

29 H2O2 in methanol at room temperature (RT). To

30 counter antigen masking, due to the formalin

31 fixation, the tissue was pretreated with microwave

32 irradiation to promote antigen retrieval. The

1   sections were washed in distilled water and placed

2   in 0.01M Tri-Na citrate pH7.8 and irradiated in a

3   Miele microwave oven for 6min (2x3min) at 450W (the

4   optimal toime and intensity of irradiation was

5   determined from preliminary studies). After

6   incubation in PBS containing 5% normal rabbit serum

7   for 10min at RT the sections were incubated in MQ1

8   ascites (diluted 1:50 in PBS) at 4C overnight.

9   Following 2x5min washes in PBS the sections were

10 incubated in biotinylated rabbit anti-mouse IgM

11 diluted 1:400 in PBS for 40min at RT. After further

12 washes in PBS, a streptavidin-biotin complex linked

13 to peroxidase was added to the sections and

14 incubated for 40min at RT. The peroxidase reaction

15 was developed in 0.1% diaminobenzidine in PBS

16 activated with 1% H2O2. After washing in water, the

17 sections were counterstained in haematoxylin,

18 dehydrated through graded alcohols, cleared in

19 xylene and mounted in DPX. In addition to negative

20 controls, incubated with a primary antibody raised

21 against Aspergillus niger glucose oxidase, positive

22 controls of histologically normal brain and

23 astrocytoma tissue were included with every batch.

24 cDNA Expression Library and screening.

25

26 G-CCM Cell cDNA Library Synthesis

27

28 A   Total RNA isolation from G CCM cells

29   This was performed using Tel-Test RNA Stat-60,

30 following their guidelines. Web Site

31 www.isotexdiagnostics.com/rna stat-60 reagent.html

32

1   B   mRNA Purification from Total RNA

2   This was performed using Invitrogen's FastTrack

3   2.0 Kit, following their guidelines. Web Site

4   www.invitrogen.com/content.cfm?pageid=3443&cfid=3308

5   35&cftoken=53475959#FastTrack

6

7   C   cDNA Library Synthesis from mRNA

8   This was performed using a Stratagene cDNA

9   synthesis kit (following their protocol).

10   Stratagene ZAP Express cDNA Synthesis Kit

11   Instruction Manual

12   www.stratagene.com/manuals/200403.pdf

13

14   RESULTS

15

16   Antibody Production

17

18   The fusion resulted in the production of five viable

19   antibody secreting hybridomas which screened

20   positively by immunofluorescence microscopy on

21   acetone fixed G-CCM cells. Of these, one (hybridoma

22   MQ1) was found to secrete an antibody which was

23   isotyped as a class M immunoglobulin with a kappa-

24   light chain. This antibody recognizes a cell

25   surface epitope, showing punctate labelling, on live

26   G-CCM cells. Further examination by confocal

27   microscopy confirmed the cell surface labelling of

28   live G-CCM cells and revealed the presence of an

29   intra-cellular epitope in permeabilized cells

30   (Figure 1A&B). In addition examination of the

31   permeabilized cells demonstrated localisation of

32   labelling at focal adhesion points on the cell

1 surface.

2

3 <u>Immunocytochemistry</u>

4

5 A range of cell lines was examined by indirect

6 immunofluorescence for the presence of the MQ-1

7 antigen

8 (Table 1).

9

<u>Table 1</u>

CELL LINE	TISSUE SOURCE	MQ1 LABELLING
Fibroblasts	Normal skin	—
C6	Rat glioma	—
FA 10 weeks	Human fetal astrocytes	+
FA 12 weeks	Human fetal astrocytes	+
FA 14 weeks	Human fetal astocytes	+
FA 15 weeks	Human fetal astrocytes	+
FA 16 weeks	Human fetal astrocytes	+
FA 19 weeks	Human fetal astrocytes	+
NP 527/94	Pilocytic astrocytoma (I)	++
NP 396/94	Pilocytic astrocytoma (I)	++
NP 424/94	Astrocytoma (II)	++
NP 676/92	Astrocytoma (II)	++
NP 445/92	Astrocytoma (II)	++
NP 204/92	Astrocytoma (II)	++
NP 482/96	Astrocytoma (II)	++
NP 473/92	Anaplastic astrocytoma (III)	+++
G-CCM	Anaplastic astrocytoma (III)	++++
NP 493/94	Anaplastic astrocytoma (III)	+++
NP 785/96	Anaplastic astrocytoma (III)	+++

NP 402/93	Glioblastoma multiforme (IV)	++++
NP 293/96	Glioblastoma multiforme (IV)	+++
NP 602/91	Glioblastoma multiforme (IV)	++++
NP 536/94	Glioblastoma multiforme (IV)	+++
NP 306/92	Glioblastoma multiforme (IV)	++++
NP 479/95	Glioblastoma multiforme (IV)	+ + +
NP 770/96	Glioblastoma multiforme (IV)	+ + +
NP 876/96	Glioblastoma multiforme (IV)	+ + + +
NP 39/96	Glioblastoma multiforme (IV)	+ + +
CB 109	Glioblastoma multiforme (IV)	—
NP 670/92	Glioblastoma multiforme (IV)	—

1   Table 1.   Indirect immunofluorescence on a range of

2   live cell lines and cell cultures with MQ1 antibody.

3

4   The results show that the human skin fibroblasts and

5   the C6, rat glioma, cell lines do not express the

6   antigen.   The fetal astrocytes and glioma cell lines

7   were positive with the exception of two cell lines

8   (CB109 and NP670/92) derived from glioblastomas

9   multiforme.   Under subjective microscopic analysis

10 there appeared.to be a variation in labelling

11 intensity between the positive cell lines.   The high

12 grade gliomas had a higher labelling intensity than

13 low grade gliomas and fetal astrocytes.   This was

14 confirmed by flow cytometry (Figure 2).   The results

15 show a progressive increase in MQ-1 antigen

16 expression, as estimated by the mean channel

17 fluorescence, from low to high grade astrocytomas,

18 the expression on grade IV astrocytomas being more

19 than double that of grade I astrocytomas.   The fetal

20 astrocytes showed a lower expression than the

21 astrocytoma cell lines, that halved from fetal

22 astrocytes of 12 weeks gestation to 16 weeks

23 gestation.

24

25 Immunohistochemistry

26

27 The results of the immunohistochemical study on

28 primary brain tumours are summarized in Table 2.

29

Table 2

Tumour	# Biopsies	MQ1 positivity
Astrocytomas	30	29/30
Neurinoma	3	0/3
Oligodendroglioma	3	0/3
Medulloblastoma	3	0/3
PNET	3	0/3

1   Table 2 Immunohistochemical analysis of MQ1 immuno-
2   labelling of a range of Primary Brain Tumours
3   showing that of the tumour tissue tested only
4   astrocytomas displayed MQ1 positivity.
5
6   The results show that of all the primary brain
7   tumours tested (oligodenrogliomas, PNET etc) only
8   astrocytomas were MQ1 positive.
9   All pilocytic (grade I) astrocytomas showed a
10 similar staining pattern. There was strong cellular
11 immunostaing of MQ1 proteins which extended to the
12 cellular processes of bipolar cells (Fig3A). The
13 immunopositive cells stood out prominently against a
14 loosely arranged less cellular stroma.
15 The astrocytomas (grade II) and anaplastic (grade
16 III) astrocytomas revealed a diffuse
17 immunopositivity and the staining pattern was
18 similar in all (Fig 3B&C). There was variation in
19 the staining pattern of glioblastomas. Out of 16
20 glioblastomas tested, 1 was unreactive revealing no
21 MQ1 protein expression whereas 14 showed focal
22 positivity and one diffuse immunostaining (Fig 3D).
23 Focal positivity was observed as clusters or groups

 1   of positive cells surrounded by unreactive aresas.

 2   Tumour cells palisading around areas of necrosis, a

 3   characteristic feature of glioblastomas also reveled

 4   focal positivity. However tumour giant cells,

 5   bizarre cells and clusters of proliferating

 6   endothelial cells were negative for MQ1 protein

 7   expression. The oligodendroglial cells were

 8   negative. Within adjacent grey matter the neurones

 9   did not show immunolabelling for the MQ1 proteins.

10   The endothelial cells lining small and large blood

11   vessels in and around tumours of all grades showed

12   no MQ1 protein expression. There was no

13   immunolabelling of lymphocytes in the perivascular

14   spaces. The infiltrating edge of the tumours and

15   the adjacent glial areas showed prominent labelling

16   of large reactive astrocytes (Fig 4 A&B)). Such

17   cells revealed multiple processes. However this MQ1

18   positivity in reactive astrocytes was only found

19   surrounding MQ1 positive tumours, other reactive

20   tissue such as MS tissue that shows prominent

21   reactive astrocytes when labeled for GFAP (FIG 4C)

22   displayed no MQ1 positivity in the 10 biopsies

23   tested (Fig 4D).

24   In non-CNS tissue tested malignant melanoma and

25   breast 2o to the brain were found to express the MQ1

26   proteins (Table 3).

Table 3

Tissue	# Biopsies	MQ1 Positivity
Breast 20 (brain)	3	3/3
Breast 1o	228	137/228

Fibroadenoma	5	0/5
Fibrocystic Diease	5	0/5
M.Melanoma2o (brain)	4	4/4

Table 3 Immunohistochemical MQ1 immunolabelling of a range of non-CNS tumours, showing MQ1 positivity in 60% of primary breast tumours and no positivity in fibrocystic diease and fibroadenomas that are non-malignant breast conditions.

Of the primary breast tumours tested 137/228 were MQ1 positive while fibrocystic diease and fibroadenoma tissues, both premalignant conditions displayed no MQ1 positivity. Figure5 shows strong MQ1 positivity in invasive ductal carcinoma cells and lobular carcinoma cells surrounded by MQ1 negative stroma.

Isolation of MQ-1 Clones

Screening of a cDNA expression library (from G-CCM mRNA) with the MQ1 antibody identified two clones with significant homology to the Jagged 1 protein (Sequence ID No's 1 and 2).

Antisense Treatment Protocol

Antisense Oligonucleotide
5'-tgg gga acg cat cgc tgc-3'   (Sequence ID No. 4)

Antisense Control Oligonucleotide
5'-tgg gga ccg cat cgc tgc-3'   (Sequence ID No. 5)

1

2 The PTO linked antisense oligonucleotide was

3 designed against the transcription initiation site

4 and kozac sequence at the beginning of the Jagged1

5 gene (Accession number AF028593). The control

6 oilgonucleotide was the same 18 mer with one base

7 changed (therefore being the tightest control

8 possible to generate). Both oligonucleotides were

9 synthesized by MWG Biotech. For colony count assays

10 G-CCM cells were seeded out into 24well plates at

11 50,000 cells/well. The cells were incubated for

12 24hrs in growth medium and then washed with serum

13 free medium (SFM). The cells were then either

14 treated with lipofectin (Invitrogen Life

15 Technologies) alone following the standard protocol

16 (at 5µl/ml) or lipofectin with the antisense and

17 antisense control oligonucleotides at a range of

18 concentrations (0.1, 0.5 and 1.0 µM) for 16hrs.

19 Following treatment the cells were washed twice with

20 SFM and then incubated in growth medium for 24 and

21 48hrs. The results (Figure 6) show that treatment

22 with the antisense oligonucleotide at concentrations

23 of 0.5 and 1.0 µM reduced the tumour cell population

24 when compared to the control oligonucleotide and

25 lipofectin alone treatment. To assess whether this

26 was due to the induction of apoptosis similarly

27 treated cells were harvested for their protein and

28 examined for Parp cleavage (an indicator of

29 apoptosis) by immunoblotting. The results (Figure

30 7) clearly show a reduction in the level of Parp at

31 0.5 and 1.0µM antisense oligonucleotide treatment

32 when compared to control oligonucleotide and

1   lipofectin alone treatment. Thus indicating that
2   the antisense oligonucleotide treatment induces
3   apoptosis in the G-CCM cells. To confirm this,
4   treated G-CCM cells were also examined for the
5   presence of cleaved Caspase 3 (another indicator of
6   apoptosis) by immunocytochemistry. The results
7   (Figure 8) show that G-CCM cells treated with 1.0μM
8   displayed caspase 3 cleavage thus indicating that
9   apoptosis was being induced. To demonstrate that
10 these effects were due to the knocking out of the
11 MQ1 proteins by the antisense oligonucleotides,
12 treated cells were examined for the presence of the
13 MQ1 proteins by immunocytochemistry with the MQ1
14 antibody. The results (Figure 9) show that the
15 expression levels of the MQ1 proteins is reduced by
16 antisense oligonucleotide treatment when compared to
17 the control oligonucleotide.
18
19 The invention described herein has potential uses as
20 a:
21
22 Diagnostic Tool- The antibody clearly distinguishes
23 astrocytomas from other primary brain tumours,
24 normal cells and reactive gliosis. In addition it
25 recognizes 60% of primary breast tumours tested.
26
27 Targeting Device- The specificity of the antibody
28 means it can be used as a targeting device such as
29 in radioimmunotherapy.
30
31 Therapeutic Target- The antibody itself can be used
32 as a therapeutic agent by blocking out signaling

1   through the MQ1/Notch pathway thus inducing

2   apoptosis in astrocytoma cells.

3

4   The invention is not limited to the embodiments

5   hereinbefore described which may be varied without

6   departing from the spirit of the invention.